Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 3449–3454, March 1998
Biochemistry

$IPF_{2\alpha}$-I: An index of lipid peroxidation in humans

DOMENICO PRATICÒ*†, ORLA P. BARRY*†, JOHN A. LAWSON*, MUSTAFA ADIYAMAN‡, SEONG-WOO HWANG‡, SUBHASH P. KHANAPURE‡, LUIGI IULIANO§, JOSHUA ROKACH‡, AND GARRET A. FITZGERALD*§¶

*Center for Experimental Therapeutics, University of Pennsylvania, Philadelphia, PA 19104-6100; and ‡Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, FL 32901

Communicated by Raymond L. White, University of Utah, Salt Lake City, UT, December 22, 1997 (received for review September 15, 1997)

ABSTRACT **Isoprostanes are prostaglandin isomers produced from arachidonic acid by a free radical-catalyzed mechanism. Urinary excretion of 8-*iso*-prostaglandin $F_{2\alpha}$, an isomer of the PGG/H synthase (cyclooxygenase or COX) enzyme product, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), has exhibited promise as an index of oxidant stress *in vivo*. We have developed a quantitative method to measure isoprostane $F_{2\alpha}$-I, ($IPF_{2\alpha}$-I) a class I isomer (8-*iso*-$PGF_{2\alpha}$ is class IV), using gas chromatography/mass spectrometry. $IPF_{2\alpha}$-I is severalfold as abundant in human urine as 8-*iso*-$PGF_{2\alpha}$, with mean values of 737 ± 20.6 pg/mg creatinine. Both isoprostanes are formed in a free radical-dependent manner in low density lipoprotein oxidized by copper *in vitro*. However, $IPF_{2\alpha}$-I, unlike 8-*iso*-$PGF_{2\alpha}$, is not formed in a COX-dependent manner by platelets activated by thrombin or collagen *in vitro*. Similarly, COX inhibition *in vivo* has no effect on $IPF_{2\alpha}$-I. Neither serum $IPF_{2\alpha}$-I, an index of cellular capacity to generate the isoprostane, nor urinary excretion of $IPF_{2\alpha}$-I, an index of actual generation *in vivo*, is depressed by aspirin or indomethacin. In contrast, both serum thromboxane B_2 and urinary excretion of its 11-dehydro metabolite are depressed by the COX inhibitors. Although serum 8-*iso*-$PGF_{2\alpha}$ formation is substantially depressed by COX inhibitors, urinary excretion of the compound is unaffected. Urinary $IPF_{2\alpha}$-I is elevated in cigarette smokers compared with controls (1525 ± 180 versus 740 ± 40 pg/mg creatinine; $P < 0.01$) and is highly correlated with urinary 8-*iso*-$PGF_{2\alpha}$ ($r = 0.9$; $P < 0.001$). Urinary $IPF_{2\alpha}$-I is a novel index of lipid peroxidation *in vivo*, which can be measured with precision and sensitivity. It is an abundant F_2-isoprostane formed in a free radical- but not COX-dependent manner. Although 8-*iso*-$PGF_{2\alpha}$ may be formed as a minor product of COX, this pathway contributes trivially, if at all, to levels in urine. Urinary excretion of both isoprostanes is elevated in cigarette smokers.**

Isoprostanes are chemically stable prostaglandin (PG)-like compounds that are produced independent of the cyclooxygenase (COX) enzyme by free radical-catalyzed peroxidation of arachidonic acid (AA) *in situ* in membrane phospholipids (1). They are cleaved by phospholipases, circulate in plasma, and are excreted in urine (2). F_2-isoprostanes are isomers of $PGF_{2\alpha}$, a natural product derived from COX and PGF synthase. Depending on the site of the original arachidonyl radical formed, four groups of regioisomers of the F_2-isoprostanes may be produced upon rearrangement, oxygenation, and reduction, each of which may comprise 16 isomers. Thus, in theory, up to 64 different F_2-isoprostanes can be generated (3, 4).

Semiquantitative estimates of their generation have been based on enzyme immunoassay and gas chromatography/mass spectrometry (GC/MS), the latter using deuterated $PGF_{2\alpha}$ as internal standard (5, 6). We have previously developed a specific method to measure a class IV F_2-isoprostane, 8-*iso*-$PGF_{2\alpha}$, using an $^{18}O_2$-labeled internal standard (7). Although this compound is produced *in vitro* and *in vivo* via free radical peroxidation of AA, we have shown that 8-*iso*-$PGF_{2\alpha}$ is also a minor product of platelet COX-1 and monocyte COX-2 dependent metabolism of AA *in vitro* (7, 8). It may also be formed in a COX-dependent manner in serum *ex vivo* (4, 9). However, COX-dependent formation seems to contribute little, if at all, to levels of 8-*iso*-$PGF_{2\alpha}$ measured in the urine of cigarette smokers (9), a syndrome of COX-1 activation (10).

Given that our original data suggest that 8-*iso*-$PGF_{2\alpha}$, but not other F_2-isoprostanes, might be formed also by a COX-dependent mechanism (7), we have developed a specific GC/MS assay for $IPF_{2\alpha}$-I, a structurally distinct (class I) F_2-isoprostane (11). We demonstrate that $IPF_{2\alpha}$-I is formed coincidental with conventional markers of lipid peroxidation in low density lipoprotein (LDL) oxidized by copper *in vitro*. Although antioxidants prevent formation of either isoprostane under these conditions, $IPF_{2\alpha}$-I, unlike 8-*iso*-$PGF_{2\alpha}$, is not formed in a COX-dependent manner by aggregating platelets *in vitro* or in serum *ex vivo*. $IPF_{2\alpha}$-I is excreted in human urine, where it is more abundant than 8-*iso*-$PGF_{2\alpha}$. Furthermore, COX inhibition *in vivo*, by either low dose aspirin or indomethacin, fails to suppress urinary levels of either isoprostane. These results suggest that measurement of urinary $IPF_{2\alpha}$-I represents a novel approach to the noninvasive assessment of oxidant stress in humans. These data also support the notion that COX activity contributes trivially, if at all, to urinary 8-*iso*-$PGF_{2\alpha}$ under physiological conditions.

MATERIALS AND METHODS

Reagents. $^{18}O_2$-labeled 8-*iso*-$PGF_{2\alpha}$ was prepared as previously described by Pickett and Murphy (12). $IPF_{2\alpha}$-I was synthesized as previously described (11). The tetradeutero derivative, 17,17,18,18-teradeuterated $IPF_{2\alpha}$-I, was prepared by a modified synthesis (unpublished results). *N,N*-diisopropylethylamine, pentafluorobenzyl (PFB) bromide, dodecane, *N,N'*-dicyclohexyl-carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide, copper sulfate ($CuSO_4$), and butylated hydroxytoluene (BHT) were purchased from Sigma. Bis(trimethylsilyl)trifluoroacetamide was purchased from Supelco. Pyridine was purchased from Mallinckrodt. All other solvents were obtained from Burdick and Jackson.

Isoprostane Extraction and Purification. Urine samples, 0.1 ml, were spiked with 300 pg of tetradeuterated $IPF_{2\alpha}$-I and then mixed well and allowed to equilibrate for 15 min at room temperature. The volume was brought up to 1 ml with phosphate

Abbreviations: PG, prostaglandin; COX, cyclooxygenase; SPE, solid phase extraction; GC/MS, gas chromatography/mass spectrometry; AA, arachidonic acid; Tx, thromboxane; LDL, low density lipoprotein; PFB, pentafluorobenzyl; BHT, butylated hydroxytoluene; TMS, trimethylsilyl.

†These authors contributed equally to this work.

§Present address: Department of Internal Medicine, University of Rome "La Sapienza" Rome, Italy.

¶To whom reprint requests should be addressed at: Center for Experimental Therapeutics, 905 Stellar Chance Laboratories, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6100. e-mail: garret@spirit.gcrc.upenn.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

buffer, pH 7.0. Then, 250 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide was added, and the sample was allowed to stand for 30 min at room temperature. It was then extracted on a RapidTrace (Zymark, Hopkinton, MA) solid phase extraction (SPE) workstation, using reverse phase SPE cartridges (C18 EC, 100 mg; International Sorbent Technology, Mid Glamorgan, U.K.) and the following parameters. The cartridge was conditioned with 3 ml of EtOH (2 ml/min) and washed with 0.5 ml of phosphate buffer, pH 7.0 (2 ml/min). The sample was loaded (1 ml/min) onto the cartridge, which was washed with 1 ml (pH 7.0) of buffer (2 ml/min), followed by 1 ml of 35% EtOH/pH 7.0 buffer (2 ml/min). The cartridge was then flushed three times with 5 ml of hexane (15 ml/min) followed by insufflation three times with 5 ml of air (30 ml/min). It was then eluted with 1 ml of EtOAc (1 ml/min) and dried under a stream of N_2, dissolved in 25 μl of MeOH, and applied onto a silica gel TLC plate (LK6D, Whatman). The plate was developed in 3% MeOH/EtOAc, along with a separate plate on which was spotted 2 μg of authentic $IPF_{2\alpha}$-I lactone, dipped in $CuSO_4$, and heated on a hot plate. The lactone was prepared by dissolving the standard in 25 μl of *N,N'*-dicyclohexyl-carbodiimide (10 mg/ml) and allowing it to stand for 30 min at room temperature. The appropriate zone was scraped, extracted from 100 μl of H_2O with 1 ml EtOAc, and dried under N_2. It was then dissolved in 10 μl each of methanol and 15% KOH/H_2O and allowed to stand for 30 min at room temperature. After addition of 100 μl of 1 N HCl, the sample was extracted with 1 ml of EtOAc and dried. *N,N*-diisopropylethylamine (10 μl) and 10% PFB bromide in acetonitrile (20 μl) were added and allowed to stand for 10 min. After drying, the sample was dissolved in 25 μl of MeOH and applied to another TLC plate, which was developed in EtOAc. A second plate, to identify the zone for scraping, was loaded with 2 μg of $IPF_{2\alpha}$-I PFB ester, developed alongside and visualized as above. The scrapings were extracted from water as above and dried. Pyridine and bis(trimethylsilyl)trifluoroacetamide, 10 μl each, were added to form the trimethylsilyl (TMS) ether derivative, allowed to stand for 10 min, and then were dried. The sample was dissolved in 20 μl of dodecane for GC/MS analysis.

8-*iso*-$PGF_{2\alpha}$ was measured as previously described (7). Serum thromboxane (Tx) formation, measured as its stable hydrolysis product, TxB_2, and the Tx metabolite in urine, 11-dehydro TxB_2, were prepared for analysis by GC/MS as previously described (10, 13).

Demonstration of IPF Class Selectivity. A mix of approximately 100 ng each of chemically synthesized $IPF_{2\alpha}$-I, $IPF_{2\alpha}$-II, $IPF_{2\alpha}$-III, 8-*iso*-$PGF_{2\alpha}$, and 12-*iso*-$PGF_{2\alpha}$ were dried in a microcentrifuge tube, and 100 μl of urine was added, vortexed, and allowed to stand for 15 min. It was then prepared as described above, except that the second TLC was omitted. As a control, an equivalent amount of the mix was subjected to saponification and extraction as described above and derivatized as the PFB ester, TMS ether. GC/MS analysis was as described below.

Gas Chromatography/Mass Spectrometry. A Fisons MD-800 mass spectrometer, equipped with a Fisons 8000 gas chromatograph and a Fisons AS-800 autosampler, was used for all analyses. The MS was operated in the negative ion electron capture ionization mode, using ammonia as the moderating gas. Ions monitored were *m/z* 569 and 573 for $IPF_{2\alpha}$-I and the internal standard, respectively. Other eicosanoids were analyzed as previously described (7–9). A 30-m, 0.25-mm i.d., 0.25-μm phase-thickness DB5-MS column was used with a temperature program of 1 min isothermal at 190°C followed by heating at 20°C/min to 320°C. The carrier gas was helium.

Formation of $IPF_{2\alpha}$-I During Lipid Peroxidation. To assess the time course of formation of $IPF_{2\alpha}$-I during copper-catalyzed lipid peroxidation, the following experiments were performed. The protocol was reviewed and approved by the Institutional Review Board, and informed consent was obtained from participants in the study. After an overnight fast, blood from healthy normolipemic volunteers (two males, two females, age 21–38 years) was collected, and LDL was prepared by sequential density gradient ultracentrifugation according to a previously described method that minimizes oxidation (8). Protein concentration, TBARS, lipid hydroperoxides, and water-soluble antioxidants were measured as previously described (14–17). Free and phospholipid-bound 8-*iso*-$PGF_{2\alpha}$ and $IPF_{2\alpha}$-I were extracted using 10 ml of ice-cold Folch solution (chloroform/methanol; 2:1, vol/vol). The organic phase containing the extracted lipids was dried under nitrogen; then, 5 ml of aqueous KOH (15%) was added, and the mixture was incubated at 45°C for 1 hr to effect hydrolysis and release of 8-*iso*-$PGF_{2\alpha}$ and $IPF_{2\alpha}$-I.

COX-1-Dependent Formation of Isoprostanes in Activated Human Platelets. Platelets were harvested as previously described (7). Platelet aggregation was studied in WP using a PAP-4 model Biodata aggregometer (Biodata, Hatsboro, PA), at 37°, in siliconized cuvettes with continuous stirring. Supernatants were collected and stored at −80°C until analysis in the presence of 0.01% BHT.

COX-Dependent and Independent Isoprostane Excretion in Human Urine. To investigate the possibility that COX might contribute to urinary $IPF_{2\alpha}$-I, we assessed the comparative effects of two distinct COX inhibitors, aspirin and indomethacin, on urinary excretion of both $IPF_{2\alpha}$-I and 8-*iso*-$PGF_{2\alpha}$. Both isoprostanes were also measured in serum to assess the capacity for a COX-dependent contribution to their formation. Eight apparently healthy volunteers were enrolled in the study (four males and four females, age 21–38 years), which was scrutinized and approved by the Institutional Review Board of the University of Pennsylvania. Informed consent was obtained from all subjects. They were all nonsmokers, and they did not take any drugs or vitamin supplements for 4 weeks before the study. Samples (urine, plasma, serum) were obtained at 10 and 5 days before aspirin and immediately before (day 0) and after 10 days treatment with 100 mg aspirin/day. Following a 2-week washout period, samples were obtained after acute (2 hr) dosing with 50 mg of indomethacin. Six-hour urine collections were obtained, and following the addition of 0.01% BHT as an antioxidant, a 50-ml aliquot was stored at −80°C until analysis. Blood was withdrawn, without stasis, from an antecubital vein using a 21G needle into a syringe containing 3.8% sodium citrate.

Studies of Isoprostane Stability in Human Urine. Urine specimens (n = 3) were analyzed immediately after collection. They were also studied after 2, 4, and 10 days at room temperature, at +4°C, at −20°C, or at −80°C, to investigate the possibility that the compounds could be generated by autooxidation. Furthermore, 10 μg of [$^8H_2$]arachidonic acid was added to each sample to evaluate any artifactual formation of the isoprostanes during sample processing.

Cigarette Smoking. To address the hypothesis that urinary $IPF_{2\alpha}$-I would be elevated in a human syndrome of oxidant stress, we studied cigarette smokers. Six healthy male volunteers who had been smoking 20–30 cigarettes per day and six nonsmoking healthy controls collected 6-hr urines for isoprostane analysis. The volunteers were aged 31–45 years and abstained from all medication for at least 2 weeks prior to the collection.

Statistical Analysis. Data are presented as the mean ± SEM. Statistical analysis was performed using analysis of variance followed by the Student's t test when appropriate. Differences were considered significant when $P < 0.05$.

RESULTS

Assay Development. $IPF_{2\alpha}$-I was prepared as previously described (11). The tetradeutero analog 17,17,18,18-d_4-$IPF_{2\alpha}$-I was prepared as described for the parent compound. It is approximately 93% pure, with the remainder being an unidentified isomer that elutes slightly later on the GC. The "blank" of the internal standard, i.e., the amount of unlabeled contaminant, is less than 1%. A representative selected ion-monitoring chromatogram for $IPF_{2\alpha}$-I in human urine is depicted in Fig. 1. To

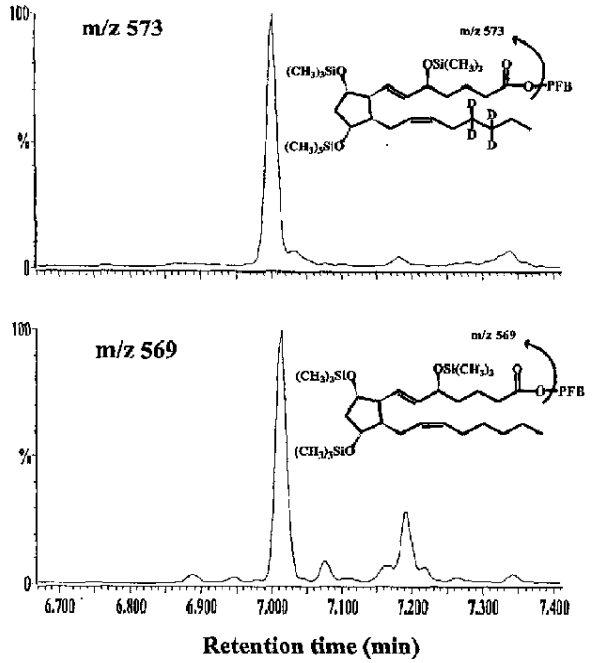

FIG. 1. Selected ion monitoring chromatogram of [$^2H_4$]IPF$_{2\alpha}$-I (*Upper*), and a comigrating peak corresponding to the endogenous compound (*Lower*), both derivatized as the PFB ester, TMS ether. The trace is representative of urinary samples from healthy volunteers and covers the entire elution range of IPF isomers.

assess the reproducibility of the IPF$_{2\alpha}$-I assay, 100-μl replicates of urine from three individuals were assayed six times on each of 3 days. The mean ± SEM values were 738.0 ± 17.2, 726.7 ± 26.6, and 748.6 ± 10.7 pg/mg creatinine. The overall mean ± SEM for all the sample analyses (n = 18) was 737.1 ± 20.6 pg/mg creatinine. The assay was linear over the range of 20–640 pg of authentic IPF$_{2\alpha}$-I to 100-μl urine aliquots. The ability of the SPE and TLC components of the assay to separate IPFs of class I from representatives of class II, III, and IV is shown in Fig. 2. Although approximately 45% of IPF$_{2\alpha}$-I was present after lactonization, SPE, TLC, saponification, and derivatization as the PFB ester, TMS ether, only about 0.04% of the other classes were present; this represents an enrichment of over 3 orders of magnitude. This selectivity takes place mainly on the TLC, where the R_f of IPF$_{2\alpha}$-I lactone is 0.2; that of classes I, II, and IV, as well as unlactonized class I compounds, is <0.02.

COX Inhibition of Isoprostane Formation. Urinary 8-*iso*-PGF$_{2\alpha}$ was measured in aliquots obtained in two different days (days −10 and −5) in untreated volunteers. The mean values were 93 ± 10 and 98 ± 12 pg/mg creatinine (Fig. 3*A*). The data ranged from 75 to 150 pg/mg creatinine and the intra- and intersubject coefficients of variation were ±5% and ±6%, respectively. To assess the effects of COX inhibition, 100 mg of aspirin was administered daily for 10 days. Efficient COX inhibition was demonstrated by suppression of urinary 11-dehydro TxB$_2$ from pretreatment values of 485 ± 12 and 500 ± 15 to 37.5 ± 2 pg/mg creatinine (P < 0.001) (Fig. 3*C*). Despite this, urinary 8-*iso*-PGF$_{2\alpha}$ on aspirin treatment (105 ± 10 pg/mg creatinine) did not differ from pretreatment levels. Following a period of washout to allow recovery of the aspirin effect, COX inhibition was again assessed by administration of indomethacin. Again, urinary 11-dehydro-TxB$_2$ was suppressed (38 ± 2.7 pg/mg creatinine), but urinary 8-*iso*-PGF$_{2\alpha}$ (103 ± 6 pg/mg creatinine) was unaltered from control values. These data indicate that COX inhibition *in vivo* in volunteers does not alter urinary 8-*iso*-PGF$_{2\alpha}$.

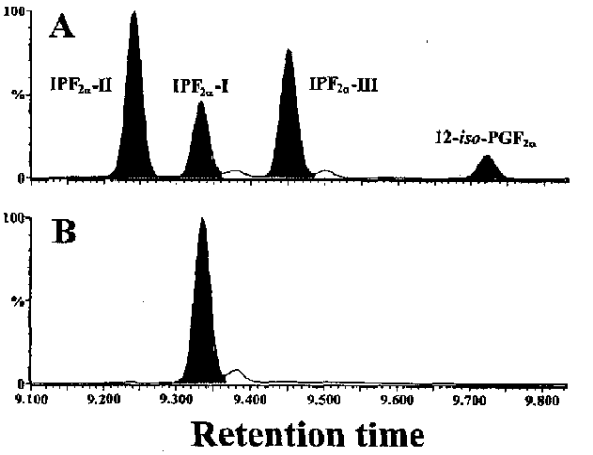

FIG. 2. Specificity of series IPF isolation. (*A*) A mixture of synthetic IP standards, IPF-I, IPF$_{2\alpha}$-III, and 12-*iso*-PGF$_{2\alpha}$ (a series IV IPF) analyzed by GC/MS. (*B*) The same mix was added to 100 μl of urine and subjected to lactonization, SPE, TLC, saponification, and derivatization are as described in the text.

The effect of COX inhibition on urinary IPF$_{2\alpha}$-I excretion was also negative. Urinary levels of the two control days were 781 ± 66 and 770 ± 70 pg/mg creatinine and ranged from 650 to 950 pg/mg creatinine. The intra- and intersubject variation in excretion was ±5% and ±4%. Levels were considerably (P < 0.0001) higher than those of 8-*iso*-PGF$_{2\alpha}$. Again, neither aspirin administration (789 ± 50 pg/mg creatinine) nor indomethacin dosing (769 ± 58 pg/mg creatinine) significantly depressed urinary IPF$_{2\alpha}$-I, despite the effects of these regimens on urinary thromboxane metabolite excretion (Fig. 3*B*). Similar results were ob-

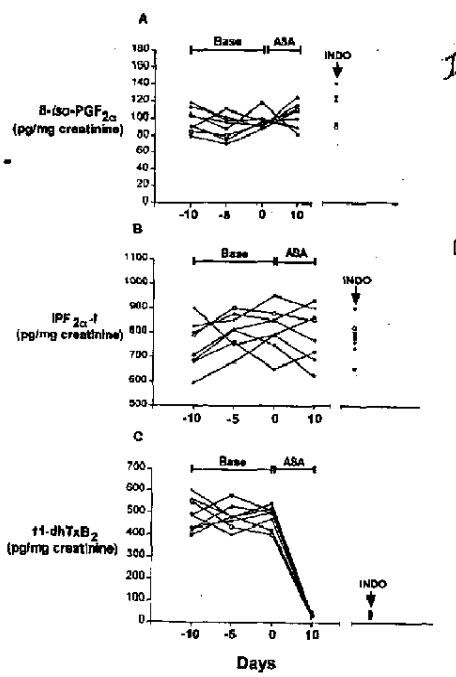

FIG. 3. Urinary excretion of 8-*iso*-PGF$_{2\alpha}$ (*A*), IPF$_{2\alpha}$-I (*B*), and 11-dehydro thromboxane B$_2$ (*C*) at 10 and 5 days before aspirin (−10, −5), immediately before aspirin (day 0) (Base), and after 10 days of aspirin intake (ASA). Following a 2-week washout, samples were collected after 2-hr indomethacin (INDO) intake.

tained when the isoprostanes were measured in plasma. Thus, neither 8-*iso*-$PGF_{2\alpha}$ (19.6 ± 2 versus 20 ± 1.5 pg/ml) nor $IPF_{2\alpha}$-I (80 ± 4.2 versus 78.5 ± 7.9 pg/ml) was suppressed by aspirin or indomethacin (21 ± 1.4 and 77.1 ± 6.8 pg/ml, respectively).

To assess the influence of COX inhibition on the capacity to generate these compounds rather than on their actual generation *in vivo*, we measured 8-*iso*-$PGF_{2\alpha}$, $IPF_{2\alpha}$-I, and TxB_2 in serum. Both the aspirin regimen (196 ± 6 versus 18 ± 2.4 ng/ml) and indomethacin (15 ± 1.5 ng/ml) significantly depressed serum levels of TxB_2, the hydrolysis product of TxA_2 (Fig. 4*C*). Although much less of the compound was formed in serum than TxB_2, depression of serum 8-*iso*-$PGF_{2\alpha}$ by both aspirin (228 ± 18 versus 28.2 ± 2 pg/ml) and indomethacin (26.6 ± 2.4 pg/ml) was also significant (Fig. 4*A*). These results indicate the capacity for COX-dependent formation of minor amounts of this compound, consistent with our previous observations (7). In contrast to our findings with 8-*iso*-$PGF_{2\alpha}$, neither aspirin (80 ± 2.2 versus 74.5 ± 5.5 pg/ml) nor indomethacin (80 ± 2.2 versus 79 ± 3.7 pg/ml) depressed serum $IPF_{2\alpha}$-I (Fig. 5*B*), suggesting that blood cells do not express the capacity to form this compound in a COX-dependent manner.

To investigate the effects of COX activation on the formation of the two isoprostanes in more detail, we studied platelet activation *in vitro*. Human platelets possess the COX-1 isoform (18), and we have previously demonstrated that they exhibit the capacity to generate 8-*iso*-$PGF_{2\alpha}$ as a minor COX product (7). Again, activation of platelets with either thrombin or collagen markedly increased 8-*iso*-$PGF_{2\alpha}$ formation. This response was abolished by pretreatment of the platelets with the COX inhibitor, aspirin (100 μM), but not by the antioxidant BHT (20 μM) (Fig. 5). $IPF_{2\alpha}$-I, by contrast, was unaltered by platelet COX-1 activation.

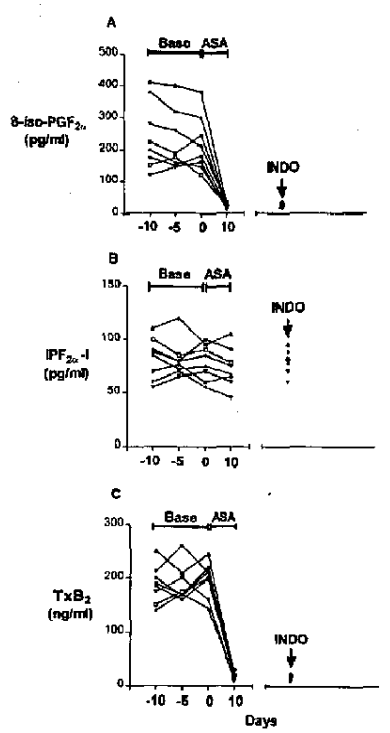

FIG. 4. Serum levels for 8-*iso*-$PGF_{2\alpha}$ (*A*), $IPF_{2\alpha}$-I (*B*), and thromboxane B_2 (*C*) at 10 and 5 days before aspirin (−10, −5), immediately before aspirin (day 0) (Base), and after 10 days of aspirin intake (ASA). After a 2-week washout, levels were measured after 2-hr indomethacin (INDO) intake.

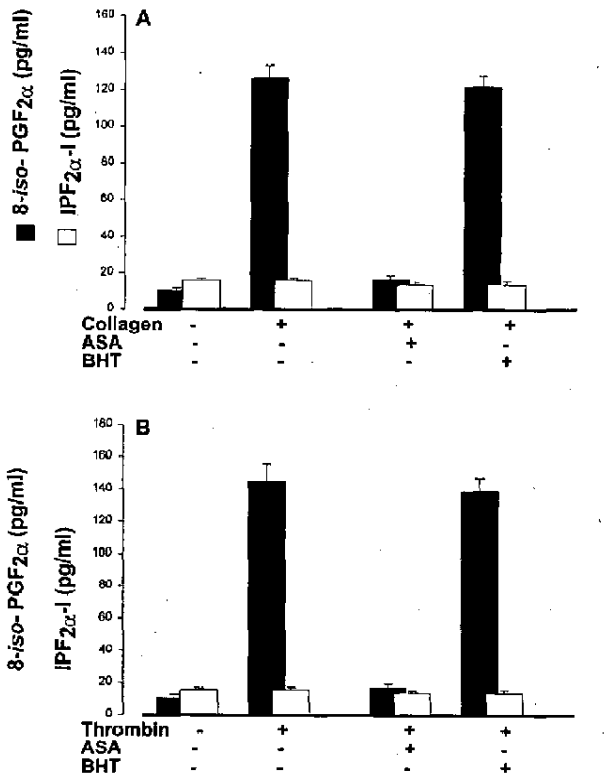

FIG. 5. 8-*iso*-$PGF_{2\alpha}$ and $IPF_{2\alpha}$-I levels during platelet aggregation induced by collagen (1 μg/ml) (*A*) or thrombin (0.1 unit/ml) (*B*). Supernatants were collected before and 3 min after adding the agonist, in the presence of vehicle, aspirin (ASA) (100 μM) or the antioxidant, butylated hydroxytoluene (BHT) (20 μM) (n = 4 for each panel).

We also investigated whether the two F_2-isoprostanes could be formed *ex vivo* by autooxidation in urine. We found that the amounts in urine obtained from normal volunteers were not increased by leaving the specimens at room temperature for up to 10 days. No difference was also observed when these levels were compared with those obtained in specimens stored at 4°C, −20°C, or −80°C for the same period (Table 1). Moreover, no artifactual formation of the isoprostanes was detected during the sampling and processing, as no deuterated form of either of the two isoprostanes was found (data not shown).

LDL Peroxidation. The time course of LDL peroxidation in response to $CuSO_4$ (10 μM) was assessed in samples from four normal volunteers (two males, two females, age 21–38 years). Total 8-*iso*-$PGF_{2\alpha}$ levels were 0.35 ± 0.04, 1.10 ± 0.08, 1.14 ± 0.09, 3.63 ± 0.18, 5.56 ± 0.31, and 8.45 ± 0.38 ng/mg LDL protein at 0, 1, 3, 6, 9, and 24 hr after addition of copper, respectively. In the same samples, $IPF_{2\alpha}$-I levels rose from 1.1 ± 0.1 to 27.8 ± 1.1 ng/mg LDL protein at 24 hr. In both cases, pretreatment with the antioxidant, BHT (20 μM), prevented the formation of both 8-*iso*-$PGF_{2\alpha}$ and $IPF_{2\alpha}$-I. Thus, both isoprostanes were formed in a free radical-dependent manner in this system. More conventional indices of lipid peroxidation, such as TBARS and lipid hydroperoxides, closely correlated with $IPF_{2\alpha}$-I in oxidized LDL (Table 2). Incubation of LDL with $CuSO_4$ (10 μM) caused the endogenous antioxidants α-tocopherol, β-carotene, and lycopene to decrease significantly (Table 2). This was followed by consumption of lutein/zeaxanthine and cryptoxanthine (data not shown).

Cigarette Smoking. Both urinary $IPF_{2\alpha}$-I (1525 ± 180 versus 740 ± 40 pg/ng creatine) and 8-*iso*-$PGF_{2\alpha}$ (270 ± 36 versus 95 ±

n=6

smoker: n=6
non-smoker: n=6

Table 1. Isoprostane stability in urine

	8-*iso*-$PGF_{2\alpha}$, pg/mg creatinine			$IPF_{2\alpha}$-I, pg/mg creatinine		
	Day 2	Day 4	Day 10	Day 2	Day 4	Day 10
RT	95.5 ± 5.5	103 ± 6.5	94 ± 5	745 ± 25	755 ± 28	760 ± 38
+4°C	97.0 ± 5	95.5 ± 5.5	101 ± 4	759 ± 40	755 ± 25	740 ± 28
−20°C	93.5 ± 3.5	98 ± 5.2	96 ± 6.3	766 ± 26	747 ± 27.3	757 ± 23
−80°C	100 ± 6	93.5 ± 3.9	94 ± 5	735 ± 36	770 ± 38	756 ± 32

Six-hour urine specimens were collected from three apparently healthy individuals. Aliquots (50 ml) were kept at different conditions for different periods of time (2, 4, and 10 days). Isoprostane levels were measured as described in *Methods*. Results are expressed as mean ± SEM. RT, room temperature.

10 pg/mg creatine) were markedly elevated in the smokers compared with the nonsmoking controls. Excretion of both isoprostanes was highly correlated ($r = 0.9$; $P < 0.001$).

DISCUSSION

Isoprostanes are non-COX-derived prostaglandin isomers, products of arachidonic acid which result from free radical-catalyzed lipid peroxidation (19, 20). They are formed initially esterified in membrane phospholipids, from which they are presumptively cleaved by a phospholipase A_2 (1), circulate in plasma, and are excreted in urine (2). Given their chemical stability, their formation by a specific, free radical-catalyzed pathway and the potential precision and sensitivity of their measurement in biological fluids by mass spectrometry (7), they seem to offer promise as indices of free radical generation in integrated systems. The present confusion as to the role of oxidant stress in human disease, the indirect approaches to determination of an antioxidant dose of common vitamins in humans (21–24), and the conflicting data emanating from clinical trials of such vitamins (25–27) suggest that the validation of such an index of oxidant stress *in vivo* is timely.

Several approaches to the measurement of isoprostanes have been adopted. These include the estimation of "total" F_2-isoprostanes, using the prostaglandin, $PGF_{2\alpha}$ as an internal standard, the use of immunoassays directed at specific compounds, and the measurement of specific isoprostanes using GC/MS. Given that up to 64 such isomers in 4 structural classes may be formed from peroxidation of arachidonic acid (3, 4), it would seem judicious at this point to measure specific compounds. Taking this approach, we developed a method to measure 8-*iso*-$PGF_{2\alpha}$, reportedly an abundant F_2-isoprostane in humans (28) and one which exhibits biological actions *in vitro*. 8-*iso*-$PGF_{2\alpha}$ modulates platelet function, constricts vascular smooth muscle, and may act as a mitogen (29–31), effects which are blocked by pharmacological antagonists of thromboxane receptors. It is unknown whether these effects have any relevance to its role *in vivo* and, if so, whether they are transduced via thromboxane receptors or related receptors (32). Measurement of 8-*iso*-$PGF_{2\alpha}$ in urine offers promise as a noninvasive approach to measurement of oxidant stress *in vivo* (33). Thus, excretion is increased dose-dependently by cigarette smoking and falls upon quitting (9). Similarly, excretion increases during coronary reperfusion after preceding ischemia in animal models and in humans (34, 35). Both cigarette smoking (36, 37) and ischemia-reperfusion (38, 39) are thought to be models of oxidant stress in humans.

However, when we turned our attention to the mechanisms of cellular formation of 8-*iso*-$PGF_{2\alpha}$, our results were surprising. Thus, although it could be generated in a free radical-dependent manner (8), 8-*iso*-$PGF_{2\alpha}$ was also a minor product of COX-1 in activated human platelets and of COX-2 in human monocytes (7, 8). These results were confirmed by others in human monocytes and in rat mesangial cells (40,41). Given that COX activation and oxidant stress might coincide in clinical settings such as smoking, ischemia-reperfusion, and inflammation, such observations questioned the usefulness of urinary 8-*iso*-$PGF_{2\alpha}$ as an index of oxidant stress *in vivo*.

To address this issue, we took two approaches. The first was to assess the effects of COX inhibition on the capacity of blood cells to form 8-*iso*-$PGF_{2\alpha}$, as reflected by measurements in serum (9), and on actual *in vivo* generation of the compound, as reflected by its excretion in urine. Inhibition of platelet COX-1 did not depress urinary 8-*iso*-$PGF_{2\alpha}$, despite a reduction in serum 8-*iso*-$PGF_{2\alpha}$ and inhibition of COX, as reflected by suppression of serum TxB_2 and excretion of urinary 11-dehydro TxB_2 (9). Similarly, we have found that controlled administration of bacterial lipopolysaccharide results in a major increment in prostaglandin metabolite excretion in urine and regulated expression of COX-1 and COX-2 *ex vivo* in volunteers (42). Lipopolysaccharide administration also increases urinary 8-*iso*-$PGF_{2\alpha}$, which, unlike the prostaglandin metabolites, is not suppressed by COX inhibitors (B. McAdam and G.A.F., unpublished data). These data suggest that although the capacity for COX-dependent formation of 8-*iso*-$PGF_{2\alpha}$ may be expressed *ex vivo*, this pathway contributes in an undetectable manner to urinary levels of the compound, even in syndromes of COX induction.

The second approach that we have adopted is illustrated in this communication. Initial observations (7, 43) and theoretical considerations (44) suggested that 8-*iso*-$PGF_{2\alpha}$ was unusual among F_2-isoprostanes in being formed by COX. To address this possibility, we synthesized an internal standard for a structurally distinct isoprostane, $IPF_{2\alpha}$-I. This compound is a member of class I F_2-isoprostanes as distinct from 8-*iso*-$PGF_{2\alpha}$ (class IV). Class I IPFs, by virtue of the C5 hydroxyl group, are able to form a cyclic lactone. Lactonization imparts to them a distinctly less polar nature and enables this class to be conveniently separated from IPFs of classes II, III, and IV by a wide margin, using TLC. We now report that $IPF_{2\alpha}$-I is not only present in human urine but more abundant than 8-*iso*-$PGF_{2\alpha}$. We demonstrate its formation,

Table 2. $CuSO_4$-dependent LDL oxidation

	0 hr	0.5 hr	1 hr	3 hr	6 hr	24 hr
TBARS (nmol/mg LDL)	0.45 ± 0.1	1.2 ± 0.2	5.2 ± 0.4	10.4 ± 0.35	17.6 ± 0.5	27.0 ± 0.45
Hydroperoxides (nmol/mg LDL)	46.0 ± 15	58.0 ± 18	128.0 ± 25	200 ± 20	400 ± 22	590 ± 35
$IPF_{2\alpha}$-I (ng/mg LDL)	1.0 ± 0.1	1.3 ± 0.09	2.15 ± 0.1	3.6 ± 0.25	9.8 ± 0.4	27.8 ± 0.85
α-Tocopherol (%)	100	51	ND	ND	ND	ND
β-Carotene (%)	100	62	23	ND	ND	ND
Lycopene (%)	100	55	19	ND	ND	ND

LDL (0.4 mg protein/ml) was incubated with 10 μM $CuSO_4$ at 37°C under air for 0–24 hr. Aliquots were withdrawn at time intervals and analyzed for levels of TBARS, lipid hydroperoxides, and $IPF_{2\alpha}$-I. Levels of LDL antioxidants were also measured and expressed as percentage of their initial concentrations. Results are expressed as mean ± SEM of four experiments. ND, not detectable.

along with 8-*iso*-$PGF_{2\alpha}$ in LDL oxidized by copper *in vitro*. $IPF_{2\alpha}$-I is also the more abundant of the two isoprostanes formed under these conditions, and the kinetics of its formation closely mirror those of more conventional indices of lipid peroxidation, as well as consumption of endogenous antioxidants, in LDL. We find no evidence for COX-dependent formation of $IPF_{2\alpha}$-I. Thus, activation of human platelets, by thrombin or collagen, results in COX-dependent formation of 8-*iso*-$PGF_{2\alpha}$ but not $IPF_{2\alpha}$-I. Similarly, administration of the COX inhibitors, aspirin and indomethacin, partially suppresses serum 8-*iso*-$PGF_{2\alpha}$, but not $IPF_{2\alpha}$-I, *ex vivo* in the volunteers. The results with 8-*iso*-$PGF_{2\alpha}$ confirm and extend our previous observations of aspirin effects on this index of cellular capacity to form the compound in nonsmoking (5) and smoking (9) volunteers.

Despite the effects of both COX inhibitor regimens on 8-*iso* generation *ex vivo*, urinary excretion of the compound was unaffected by either aspirin or indomethacin. However, we did observe marked suppression of the 11-dehydro metabolite of thromboxane, a COX product of platelets and macrophages. Similarly, urinary $IPF_{2\alpha}$-I was unaffected by these therapeutic maneuvers. Finally, we confirmed that urinary $IPF_{2\alpha}$ is increased in cigarette smokers, a syndrome of oxidant stress *in vivo*. As expected, we confirmed that $IPF_{2\alpha}$-I was the more abundant isoprostane in the smokers' urine. Consistent with their formation by a common mechanism, urinary levels of both isoprostanes were highly correlated.

The utility of F_2-isoprostane measurement in settings of oxidative stress has now been validated by several groups. Several mass spectrometric approaches have been utilized. However, attempts to use a single internal standard to quantitate all of the isomers present in urine depend on reproducible recovery of all these species and the lack of any other compounds contributing to the ion current within the GC elution region of the F_2-isoprostanes. The former is optimized when sample purification is minimized, the latter when purification is maximized. Monitoring a single isomer is thus theoretically attractive, but purification of one isoform from the many others present results in a technically demanding and time-consuming sample preparation protocol. The task of purification of a single isomer is drastically reduced by taking advantage of the ability of the class I isoprostanes, such as $IPF_{2\alpha}$-I, to form a cyclic lactone, easily separable from the three other classes.

Our results suggest that $IPF_{2\alpha}$-I is a particularly appealing index of lipid peroxidation *in vivo*. It can be measured with precision and sensitivity, exhibits low intra- and intersubject variation in volunteers, and is more abundant than 8-*iso*-$PGF_{2\alpha}$ in human urine. Unlike 8-*iso*-$PGF_{2\alpha}$, it exhibits no capacity for COX-dependent formation and may be used to verify elevations in urinary 8-*iso*-$PGF_{2\alpha}$ in syndromes where COX induction and oxidant stress may coincide. Parenthetically, current evidence suggests that COX-dependent formation contributes trivially, if at all, to urinary excretion of 8-*iso*-$PGF_{2\alpha}$.

We thank National Institutes of Health for support from Grants MO IRR0040, HL54500, and DK44730 and a grant-in-aid from the American Heart Association. We gratefully acknowledge support from the National Science Foundation for an AMX-360 NMR instrument (CHE 9013145) and from the Turkish Ministry of Education for a doctoral fellowship (to M.A.). G.A.F. is the Robinette Foundation Professor of Cardiovascular Medicine.

1. Morrow, J. D., Awad, J. A., Boss, H. J., Blair, I. A. & Roberts, L. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10721–10725.
2. Awad, J. A., Morrow, J. D., Takahashi, K. & Roberts, L. J. (1993) *J. Biol. Chem.* **268,** 4161–4169.
3. Waugh, R. J. & Murphy, R. C. (1996) *J. Am. Soc. Mass Spectrom.* **7,** 490–499.
4. Hwang, S.-W., Adiyaman, M., Khanapure, S. P., Schio, L. & Rokach, J. (1994) *J. Am. Chem. Soc.* **116,** 10829–10830.
5. Wang, Z., Ciabattoni, G., Creminon, C., Lawson, J., FitzGerald, G. A., Patrono, C. & Maclouf, J. (1995) *J. Pharmacol. Exp. Ther.* **275,** 94–100.
6. Morrow, J. D. & Roberts, L. J. (1994) *Methods Enzymol.* **233,** 163–174.
7. Praticò, D., Lawson, J. A. & FitzGerald, G. A. (1995) *J. Biol. Chem.* **270,** 9800–9808.
8. Praticò, D. & FitzGerald, G. A. (1996) *J. Biol. Chem.* **271,** 8919–8924.
9. Reilly, M., Delanty, N., Lawson, J. A. & FitzGerald, G. A. (1996) *Circulation* **94,** 19–25.
10. Nowak, J., Murray, J. J., Oates, J. A. & FitzGerald, G. A. (1987) *Circulation* **76,** 6–14.
11. Adiyaman, M., Lawson, J. A., Hwang, S. W., Khanapure, S. P., FitzGerald, G. A. & Rokach, J. (1996) *Tetrahedron Lett.* **37,** 4849–4852.
12. Pickett, W. C. & Murphy, R. C. (1981) *Anal. Biochem.* **111,** 115–121.
13. Catella, F., Healy, D., Lawson, J. & FitzGerald, G. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5861–5865.
14. Lowry, O. H., Rosebrough, M. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
15. Iuliano, L., Praticò, D., Ghiselli, A., Bonavita, M. S. & Violi, F. (1992) *Lipids* **27,** 349–353.
16. Nourooz-Zadeh, J., Taajaddini-Sarmandi, J. & Wolff, S. P. (1994) *Anal. Biochem.* **220,** 403–409.
17. Iuliano, L., Colavita, A. R., Camastra, C., Bello, V., Quintarelli, C., Alessandroni, M., Piovella, F. & Violi, F. (1996) *Br. J. Pharmacol.* **119,** 1438–1446.
18. Funk, C., Funk, L. B., Kennedy, M., Pong, A. & FitzGerald, G. A. (1991) *FASEB J.* **5,** 2304–2312.
19. Morrow, J. D., Harris, T. M. & Roberts, L. J. (1990) *Anal. Biochem.* **184,** 1–10.
20. Delanty, N, Reilly, M., Praticò, D., Fitzgerald, D. J., Lawson, J. A. & FitzGerald, G. A. (1996) *Br. J. Clin. Pharmacol.* **42,** 15–19.
21. Princen, H. M. G., van Poppel, G., Vogelezang, C., Buytenhek, R. & Kok, F. J. (1992) *Arterioscler. Thromb. Vasc. Biol.* **12,** 554–562.
22. Reaven, P. D. & Witztum, J. L. (1993) *Arterioscler. Thromb. Vasc. Biol.* **13,** 601–608.
23. Belcher, J. D., Balla, J., Balla, G., Jacobs, D. R., Gross, M., Jacob, H. S. & Vercellotti, G. M. (1993) *Arterioscler. Thromb. Vasc. Biol.* **13,** 1779–1789.
24. Levine, M., Conry-Cantilena, C. C., Wang, Y., Welch, R. W., Washko, P. W., Dhariwal, K. R., Park, J. B., Lazarev, A., Graumlich, J. F., King, J. & Cantilena, L. R. (1995) *Proc. Natl. Acad. Sci. USA* **93,** 3704–3709.
25. Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B. & Kromhout, D. (1993) *Lancet* **342,** 1007–1111.
26. Stephens, N. G., Parson, A., Schofield, P. M., Kelly, F., Cheeseman, K., Mitchinson, M. J. & Brown, M. J. (1996) *Lancet* **347,** 781–786.
27. Rapola, J. M., Virtamo, J., Ripatti, S., Huttunen, J. K., Albanes, D., Taylor, P. R. & Heinonen, O. P. (1997) *Lancet* **349,** 1715–1720.
28. Morrow, J. D., Minton, T. A., Badr, K. F. & Roberts, L. J. (1994) *Biochim. Biophys. Acta* **1210,** 244–248.
29. Morrow, J. D., Minton, T. A. & Roberts, L. J. (1992) *Prostaglandins* **44,** 155–163.
30. Banerjee, M., Kang, K. H., Morrow, J. D., Roberts, L. J. & Newman, J. H. (1992) *Am. J. Physiol.* **263,** H660–H663.
31. Fukunaga, M., Makita, N., Roberts, L. J., Morrow, J. D., Takahashi, K. & Badr, K. F. (1993) *Am. J. Physiol.* **264,** C1619–C1624.
32. Praticò, D., Smyth, E., Violi, F. & FitzGerald, G. A. (1996) *J. Biol. Chem.* **271,** 14916–14924.
33. Patrono, C. & FitzGerald, G. A. (1997) *Arterioscl. Thromb. Vasc. Biol.* **17,** 2309–2315.
34. Delanty, N., Reilly, M., Praticó, D., Lawson, J. A., McCarthy, J., Wood, F., Ohnishi, S. T., Fitzgerald, D. J. & FitzGerald, G. A. (1997) *Circulation* **95,** 2492–2499.
35. Reilly, M., Delanty, N., Roy, L., O'Callaghan, P., Crean, P. & FitzGerald, G. A. (1997) *Circulation* **96,** 3314–3320.
36. Folsom, A. R., Wu, K. W., Rosamond, W. D., Sharrett, A. R. & Chambless, L. E. (1997) *Circulation* **96,** 1102–1108.
37. Miller, E. R., Appel, L. J., Jiang, L. & Risby, T. H. (1997) *Circulation* **96,** 1097–1101.
38. Bolli, R., Zughaib, M., Li, X.-Y., Tang, X.-L., Sun, J.-Z., Triana, J. F. & McCay, P. B. (1995) *J. Clin. Invest.* **96,** 1066–1084.
39. Sakamoto, A., Ohnishi, S. T., Ohnishi, T. & Ogawa, R. (1991) *Free Radic. Biol. Med.* **11,** 385–391.
40. Patrignani, P., Santini, G., Panara, M. R., Sciulli, M. G., Greco, A., Rotondo, M. T., Di Giamberardino, M., Maclouf, J., Ciabattoni, G. & Patrono, C. (1996) *Br. J. Pharmacol.* **118,** 1285–1293.
41. Klein, T., Reutter, F., Schweer, H., Seyberth, H. W. & Nüsing, R. M. (1997) *J. Pharmacol. Exp. Ther.* **282,** 1658–1665.
42. McAdam, B. V. & FitzGerald, G. A. (1996) in *New Targets in Inflammation,* eds. Bazan, N. & Vane, J. R. (Kluwer, London), pp. 117–123.
43. Hecker, M., Ullrich, V., Fischer, C. & Meese, C. O. (1987) *Eur. J. Biochem.* **169,** 113–123.
44. Corey, E. J., Shih, C., Shih, N.-Y. & Shimoji, K. (1984) *Tetrahedron Lett.* **25,** 5013–5016.